STUDIES IN THE METABOLISM OF PATHOGENIC ACTINOMYCETES (STREPTOTHRICES). I

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INTRODUCTION

A great deal of confusion exists on the subject of classification as well as on the biochemical activities of the actinomycetes. Different organisms have been often studied under the same name, and the same organism has been studied under different names. Not only the species have been mixed up, but investigators could not agree even on the generic name, and the names Actinomyces, Streptothrix, and Nocardia have been used indiscriminately for designating often a single species. This is due largely to the fact that morphological studies of these organisms were made almost exclusively, and since morphologically and even culturally many species largely resemble one another, the reason for confusion becomes evident. Not only the older investigators, but even the more modern, which have at their possession numerous biochemical methods that can be applied advantageously to the study of these organisms, are making errors in their attempt to classify them. For example, in the "Preliminary Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types" for 1917,[1] the actinomycetes are divided into two genera: Actinomyces, usually parasitic organisms; no aerial hyphae or conidia; Actinomyces bovis taken as type; and Nocardia, producing aerial mycelium. This classification will not hold, since many pathogenic organisms, when grown on inorganic mediums, will produce aerial hyphae and conidia, and even the type taken, namely, Actinomyces bovis, will produce a scant aerial mycelium on inorganic medium. As to the pathogenicity, there is no doubt that many pathogenic actinomycetes are isolated from the soil and are true saprophytes. The presence or absence of aerial mycelium, as attempted by the committee for generic differentiation, size and shape of spores, as attempted by Krainsky[2] and Conn,[3] for specific differen-

Received for publication, June 17, 1918.

[1] Winslow, Broadhurst, Buchanan, Rogers, and Smith: Jour. Bacteriol., 1917, 2, p. 505.
[2] Centralbl. Bakteriol., II, 1915, 41, p. 649.
[3] N. Y. Agr. Exp. Station, Bull. 60.

tiation; or, pigment production, as attempted by some of the older investigators, cannot be used as primary factors in dividing these organisms; these characters should be used only as secondary points in the differentiation of the different species. Biochemical studies of these organisms will no doubt throw a great deal of light on the nature of the organisms and thus help to bring about a more thorough classification.

The term "Actinomyces" will be applied in this paper to the pathogenic forms, both for animals and plants, as well as to saprophytic forms, from many of which the pathogenic can hardly be differentiated morphologically, culturally and chemically. A complete bibliography on the subject of the nature and activities of the actinomycetes is found in the recent papers of Krainsky,[2] Waksman and Curtis,[4] and Conn.[3] All these investigators have used, in addition to organic mediums, also inorganic mediums for the growth of the actinomycetes, and thus made an important step forward in the study and classification of this very interesting and large group of organisms. While Krainsky and Conn used largely the morphologic characters in classifying these organisms, Waksman and Curtis attempted to apply also biochemical studies. None of them have succeeded so far in offering a good basis for classifying the actinomycetes, but their work is a step forward in the direction of the proper study of the organisms. A detailed report of the biochemical activities of the actinomycetes isolated from the soil will soon appear (Waksman[5]). This paper, therefore, deals only with the pathogenic forms, plant and animal.

Organisms Studied

Actinomyces bovis Harz (Syn. Streptothrix bovis).

Actinomyces asteroides (Eppinger) Gasperini (Syn. Streptothrix Eppingeri Rossi-Doria).

Actinomyces madurae (Vincent) Lehmann and Newmann (Syn. Streptothrix madurae Vincent).

Actinomyces hominis Boström.

These four cultures were obtained from Dr. K. F. Meyer of the Hooper Foundation, University of California, who received them originally from the Pasteur Institute in Paris and the New York Museum of Natural History.

Actinomyces scabies (Syn. Oospora scabies Thaxter), isolated from several sources and received from different investigators (the potato-scab strain was used in this work).

Actinomyces psolensis Taubenhaus,[6] pathogenic to sweet potatoes and isolated also by the author from the soil. The culture of Dr. Taubenhaus was used in this work.

[4] Soil Science, 1916, 1, p. 101.
[5] Soil Science (to be published soon).
[6] Jour. Agr. Research, 1918, 13, p. 437.

METHODS

Only the analytic data are given in this paper. Although the organisms were grown successfully on a number of organic mediums only those found useful for the growth of these organisms are mentioned.

For the study of hemolysis, blood agar was used; 10% of sterile defibrinated rabbit blood were added to sterile 2.5% nutrient (sugar free) agar having a reaction of + 1.0; the agar was well mixed with the blood, then distributed into sterile tubes, which were slanted, allowed to cool, then incubated to insure sterility; 15% of Gold Label gelatin in distilled water and adjusted to neutrality was used for the study of gelatin liquefaction. ($P_H = 7.0$ or hydrogen ion concentration $= 10^{-7}$; the colorimetric method was used.) The gelatin was poured into sterile Petri dishes, these were cooled, inoculated and allowed to stay at room temperature (18-20 C.). Skimmed milk sterilized at 15 lbs. for 30 minutes was used for the study of coagulation of the milk and proteolytic action on the milk proteins. For serum liquefaction, Löffler's blood serum mixture was used. For the study of the proteolytic enzymes of the organisms, 2 c c of the culture were added to 10 c c of the 15% gelatin in distilled water or to 10 c c of sterile skimmed milk; 1 c c of toluol was added to each tube, to insure sterility. All the cultures, except the gelatin plates, were incubated at 37 C.

The proteolytic action of the actinomyces and their enzymes was followed by the determination of the amino-nitrogen of the culture, by the use of the Van Slyke apparatus.

BLOOD AGAR CULTURES

A. madurae: A good dark-brown growth was obtained in 48 hours. A sharply defined, transparent, completely hemolized zone from 2-4 mm. in width around the growth in 15 days.

A. hominis: Good growth in 24 hours. Distinct hemolysis in 4 days, 3-4 mm. clear zone in 15 days.

A. bovis: Good spreading growth, faint hemolysis; clear zone in 15 days only 1-2 mm.

A. asteroides: A thin brownish smear appears in 24-48 hours, no hemolysis in 6 weeks. Gray aerial mycelium appears in 8-10 days.

A. psolensis: Greenish fine network on surface of agar, no hemolysis in 6 weeks.

A. scabies: No growth on blood agar.

Blood agar was found to be a very good medium for actinomyces, particularly for the animal pathogens. The hemolysis is characteristic of the species, and, as will be seen later, is a property of many actinomycetes which have a strong proteolytic power. The presence or absence of hemolysis can no doubt serve as a distinguishing feature in the identification of the organisms. Aerial mycelium was produced only in the case of the A. asteroides.

BLOOD SERUM CULTURES

A. madurae: Growth in 48-72 hours; serum dissolved in 6 days around the growth.

A. hominis: Good growth in 48-72 hours; serum dissolved in 6 days around the growth.

A. bovis: Minute yellowish colonies, serum not dissolved in 6 weeks, only becoming transparent.

A. asteroides: Very good growth in 48-72 hours. Serum not dissolved in 6 weeks, has not even become transparent.

A. psolensis: Good growth, serum not dissolved.

A. scabies: Scant growth, serum not dissolved.

Löffler's blood serum mixture forms a good medium for the cultivation of many actinomycetes, particularly the forms pathogenic for animals; the liquefaction of the serum can be taken as a characteristic point in differentiating the organisms. No aerial mycelium was produced on any of the organisms, when grown on this medium. By comparing the results of the growth of the organisms, one can readily observe that the hemolysis of the blood and liquefaction of the serum run parallel and are characteristic properties of the same organisms.

GELATIN (15% IN DISTILLED WATER) CULTURES

A. madurae: Gelatin begins to liquefy rapidly (6 days) around the colony, and in 15 days (18-20 C.) the liquefied zone is 5-12 mm. wide. No pigment produced.

A. hominis: Gelatin begins to liquefy in 3-4 days, and in 15 days liquefied zone is 4-7 mm. wide. No pigment produced.

A. bovis: Gelatin does not liquefy till very late. In 15 days, there is a faint liquefied zone (¼ mm.) around the growth. No pigment produced.

A. asteroides: No liquefaction of the gelatin, even in 30 days. No pigment production. Growth present (gray, thin).

A. psolensis: Gelatin slowly liquefied; in 15 days, clear zone is 4-5 mm. wide. No pigment produced.

A. scabies: Gelatin is not liquefied in 30 days; growth very slow.

The liquefaction of gelatin, which would designate the production of a proteolytic enzyme, runs parallel with the action of the organisms on blood agar and blood serum. No aerial mycelium was produced in any of the cultures studied here; no pigmentation took place in any of the cultures reported above, although, as can be seen in another place (Waksman and Curtis[4]) this gelatin is pigmented by a number of actinomyces. Lehmann and Neumann[7] report a limited liquefaction of gelatin by A. bovis; A. madurae is reported by Vincent as not liquefying gelatin (Lehmann and Newmann); while Petruschky[8] reports that it does liquefy gelatin; A. asteroides does not liquefy gelatin, after Petruscky.

[7] Atlas and Principles of Bacteriology, Pt. II, 1901, p. 438.

[8] Handbuch der pathogenen Mikroorganismen, Kolle and Wassermann, 1913, 5, p. 267.

MILK CULTURES *

The data obtained from the growth of actinomyces on milk at 37 C. are presented in Table 1.

TABLE 1

THE GROWTH OF PATHOGENIC ACTINOMYCETES ON MILK
Total Nitrogen in 10 c c of Milk = 59.6 mg.

Name of Organism	Clot Days	Peptonization		NH_2 - N in 10 c c 15 days' incubation	Per Cent. of Total Nitrogen
		Begins, Days	Complete, Days		
Control....................	—	—	—	1.33	2.2
A. madurae................	3	4	15	35.63	59.8
A. hominis.................	6	8	15 Half pepto-nized	19.95	33.5
A. scabies..................	9	10	—	(Not deter-mined)	—
A. bovis....................	—	Digestion without previous coagulation		9.98	16.8
A. poolensis................	--	Digestion without previous coagulation		12.69	21.3
A. asteroides..............	—	–	—	5.72	9.6

Milk can be very readily used for the differentiation of the pathogenic actinomyces. The ability to coagulate and then peptonize the milk differs with the different organisms. No doubt that the acclimatization of the organisms plays an important part in this respect, as will be brought out later.[5] The most important point to be observed in this experiment is the proteolytic action of the different actinomycetes. A. madurae producing most hemolysis, being the strongest gelatin liquefier, digesting more blood serum than the others, produces also the quickest coagulation of the milk and strongest digestion of the milk proteins: nearly 60% of the proteins of the milk have been digested and converted into amino-acids and other simple nitrogenous compounds showing a large content of amino-nitrogen. A. hominis, which was found, in the previous experiments, a somewhat weaker proteolytic organism than A. madurae, clotted the milk only in 6 days and digested the milk proteins almost to a half the extent than the latter organism. A. bovis, which was found to possess still weaker proteolytic activities than the first two organisms, did not clot the milk at all, but digested it with the production of amino-nitrogen amounting to 16.8% of the total nitrogen of the milk. A. asteroides, which was found to have almost no proteolytic activities, exerted no visible action whatsoever on the milk in 15 days, at 37 C., although it made a good growth on it. When the amino-nitrogen of the milk was determined it

* On repeated inoculation some of the organisms were found to behave in a slightly different way upon milk. This will be brought out later[5] and explanations suggested.

was found to increase from 2.2-9.6% of the total nitrogen, showing that some proteolysis has taken place, although no visible change was observed. A. psolensis behaved in a manner similar to A. bovis, but producing a somewhat stronger proteolytic action. A. scabies clotted the milk in 9 days, but produced very little decomposition of the clot in 15 days.

Lehmann and Neumann stated that A. bovis does not change milk in 8 days; the milk culture used in the above experiment showed hardly any change in 8 days, but in 15 days the culture became clear, almost transparent (faint turbidity). Petruschky reported that A. madurae coagulates milk, which is later redissolved, and A. asteroides produces no coagulation of the milk, although a red-brown growth is obtained; these results are confirmed by the writer.

The reaction of the milk was tested, after the 15 day period, to see whether the clotting may not be due to acid production in the milk; with sodium alizarine sulfonate as an indicator, the medium was found to be in all cases alkaline. The question of reaction changes in culture mediums of actinomycetes as well as a more detailed study in the nitrogen and carbohydrate metabolism of these organisms will be published elsewhere.[5]

To study the production of a rennet-like and of proteolytic enzymes by the actinomycetes in milk the following procedure was used:

Two c c of the milk, on which the organisms grew for 15 days (digested or not) was added to tubes containing 10 c c of sterile skimmed milk or 10 c c of sterile gelatin (15% in distilled water, dissolved, adjusted to neutrality, filtered and sterilized for 30 minutes at 100 C. on 3 consecutive days). One c c of toluene was added to each tube to prevent any growth of the organism; the tubes were stopped with rubber stoppers, shaken, and incubated at 37 C.

The results are presented in Tables 2 and 3. As a control, the same amount of inoculum, boiled for 5 minutes, was added to the gelatin and milk, treated as stated above and also incubated at 37 C. The enzyme cultures were incubated for 5 days, then the amino-nitrogen was determined in all of them to see whether any proteolytic action has taken place. The gelatin tubes were placed for 1 hour on ice, and if the tube remained liquid, the liquefaction was taken as positive.

All the 4 strains, pathogenic for animals, seem to produce a proteolytic enzyme, when grown on milk, but the quantity of the enzyme is distinctly different for the different organisms; A. bovis and A. asteroides did not produce any rennet-like enzyme in this experiment, but the culture of the first organism contained a fairly good proteolytic enzyme and the culture of the latter a very weak enzyme or only

a trace of it; A. madurae and A. hominis produced both a rennet-like and a strong proteolytic enzyme; the action of this enzyme was different for the 2 organisms: A. madurae, being the stronger proteolytic organism, produced also a larger amount or a more active enzyme.

TABLE 2

THE ACTION OF THE ENZYMES OF ACTINOMYCETES (OBTAINED FROM 15 DAY MILK CULTURES) ON MILK

Organism	Clot Production in Days		$NH_2 - N$ mg. in 10 c c	
	Control	Enzyme	Control	Enzyme
A. madurae	—	2	7.05	13.79
A. hominis	—	2	4.43	7.76
A. bovis	—	?	2.77	8.58
A. asteroides	—	—	2.06	2.48

Similar observations can be made from Table 3.

TABLE 3

THE ACTION OF THE PROTEOLYTIC ENZYME OF ACTINOMYCETES (OBTAINED FROM 15 DAY MILK CULTURES) ON GELATIN

Organism	Gelatin Liquefaction in Days		$NH_2 - N$ mg. in 10 c c	
	Control	Enzyme	Control	Enzyme
A. madurae	—	2	10.88	15.39
A. hominis	—	2	8.27	13.11
A. bovis	—	3	6.61	10.83
A. asteroides	—	— (10 days not liquefied)	5.89	5.95

The action of the proteolytic enzymes obtained from the milk is alike on gelatin and on the milk proteins, showing that the enzyme is probably the same, particularly since the reaction of the milk and of the gelatin were the same (about neutral).

The organisms were also grown on several synthetic mediums.

A detailed discussion of these will be found elsewhere. Mention will only be made of those mediums that can be used for the growth of some pathogenic actinomycetes. Saccharose, dextrose, and glycerol synthetic agars and Krainsky's glucose agar are reported here. As a basis for the first 3 mediums the Czapek's solution agar (without the saccharose) was used. The composition of this medium is as follows:

Distilled water	1,000 c c
$NaNO_3$	2.00 gm.
$MgSO_4$	0.50 gm.
K_2HPO_4	1.00 gm.
KCl	0.50 gm.
$FeSO_4$	0.01 gm.
Agar	15.00 gm.

To this medium 30 gm. of saccharose, dextrose, or glycerol were added, the materials were dissolved by boiling and, without further adjustment of the reaction, filtered through absorbent cotton, tubed, and sterilized at 15 lbs. pressure for 30 minutes.

Krainsky's glucose agar has the following composition:

Distilled water	1,000 c c
Agar	15.0 gm.
Glucose	10.0 gm.
Asparagin	5.0 gm.
K_2HPO_4	0.5 gm.

A. madurae grows equally well (growth spreading and growing deep into the medium, scant white mycelium on saccharose and glycerol) on all the four mediums, particularly where dextrose or glycerol are present as a source of carbohydrates.

A. hominis grows very well on all the four synthetic mediums, producing a heavy white mycelium.

A. bovis grows slowly on all the four mediums, never producing any very extensive growth.

A. asteroides grows well on all the four mediums, best on the dextrose synthetic agar, least on the saccharose synthetic agar (growth particularly heavy on the Czapek's synthetic solution agar, when saccharose is replaced by dextrose).

SUMMARY

Blood agar is a very good medium for the growth of pathogenic actinomycetes, a good growth being obtained in 24-72 hours when incubated at 37 C.

The production of hemolysis of the blood on blood agar, the liquefaction of blood serum, the clotting and subsequent peptonization of the milk, the liquefaction of gelatin, run parallel; the organism that produces most hemolysis, produces liquefaction of the blood serum and gelatin and a greater digestion of the milk proteins; the organism that does not produce any hemolysis of the blood does not liquefy the blood serum and the gelatin, does not clot the milk and has only a small action on the milk proteins. These characters can be used advantageously in the identification and classification of the actinomycetes.

Some pathogenic actinomycetes grow readily on synthetic mediums.